REMARKS/ARGUMENTS

Claims 2, 5, 12, 21, and 22 are under prosecution in this application. Claim 21 has been amended to better claim the subject matter which Applicant regards as the invention. Claim 5 has been amended for improved clarity. Claim 22 has been added. Support is found throughout the Specification, particularly from page 11, line 17 to page 14, line 14, and the examples illustrated from page 16, line 1 to page 19, line 26. No new matter has been added with this Amendment.

Claim Rejections under 35 U.S.C. § 102:

Claims 2 and 21 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Liu *et al.* Applicant respectfully traverses this rejection.

The invention is a method of predicting a risk for metastasis of a prostate cancer cell by measuring the MUC18 expression by immunoassay using antibodies specific for the MUC18 polypeptide, where a higher level of MUC18 expression in the prostate cancer cell compared to that in a normal prostate cell indicates that the prostate cancer cell is likely to metastasize.

In contrast, Liu *et al.* describes studies in which the anti-HNK-1 monoclonal antibody (Mab) reactivity was measured in prostatic cancer tissues. Based on these studies, the authors concluded that "a survival rate and a longer interval free of progression were associated with a higher fraction of positively stained cancer cells".

The claimed invention is not anticipated by Liu *et al.* The antibodies used in the method of the invention are directed against peptide epitopes of the MUC18 polypeptide. Anti-HNK-1 Mabs used in Liu *et al.* were prepared against a carbohydrate antigen, not against a peptide antigen (*See* Ong *et al.* JBC [2002] 277:18182-18190 submitted herewith as Exhibit A). See also the Specification at page 2, lines 12-13, where it is stated that the "carbohydrate modification [of

MUC18] known as HNK-1 or CD57" is carried by MUC18, i.e., the carbohydrate moiety is displayed on other proteins including NCAM, the neural cell adhesion molecule. Thus, the monoclonal antibody discussed in the cited reference is not specific for the MUC18 protein and would not be expected to cross react with an epitope on the peptide component of MUC18 in prostate cancer cells. It is well documented that HNK-1 antigen is expressed in many cell types, typically in cell surface proteins (*See* Ong *et al.*). Anti-HNK-1 Mab recognizes the epitope present in the carbohydrate moiety of MUC18, not in the peptide chain of this protein.

Because of this difference, the levels of HNK-1 antigen expression measured by employing anti-HNK-1 Mabs cannot be equated with the levels of MUC18 expression measured by employing the anti-MUC18 antibodies of the present invention. This is clear from the findings reported in the cited reference; the expression of HNK-1 was decreased or absent with the increasing pathological grade of prostate cancer whereas the increased expression of MUC18 was observed in metastatic prostate cancer cells in the present disclosure.

In summary, it is submitted that claims 2 and 21 are not anticipated by Liu *et al.* Newly added claim 22 is also not anticipated by the cited reference for the same reasons. Withdrawal of the rejection under 35 U.S.C. § 102(b) is respectfully requested.

Claim Rejections under 35 U.S.C. § 112:

Claims 2, 5, 12, and 21 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the Specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Office Action specifically points out that the phrases, "for predicting a risk for metastasis" and "a MUC18 coding sequence" recited in claim 21 are not supported by the Specification. Claim 21 has been amended to recite "the MUC18 coding sequence" according to the Examiner's suggestion.

Applicant submits that the present disclosure does provide sufficient description to support the claims at issue based on the following:

1) The purpose of the present invention was to provide improved diagnostic test (i.e. method for predicting the risk for metastasis) for metastatic prostate cancer (*See* page 2, lines 17-19).

2) The reagents (antibodies) necessary for making the invention were obtained (Example 1 on page 16).

3) Using the antibodies made, expression levels of MUC18 in four cell lines were determined; the results showed that the MUC18 protein was only expressed in three metastatic prostate cancer cell lines but not in one non-metastatic cancer cell line (Fig.6).

4) Expression levels of MUC18 in normal prostatic gland, normal primary human prostate epithelial cells and tissues of a patient with malignant prostatic cancer were measured by Western blot analysis; the results were consistent with those obtained in 3) (Fig.7).

The results of the studies of 3) and 4) provided a positive correlation between the level of MUC18 expression and the pathologic grade of prostate cancer.

5) To further establish that the expression of MUC18 is correlated with the ability to metastasize, the LNCAP (non-metastatic cancer cell line) cell line was engineered to express MUC18, and the MUC18 expressing cells were examined *in vitro* for metastatic ability, i.e., motility and invasiveness. The metastatic rates were also tested *in vivo* in athymic nude mice (page 13, lines 14-29; Example 2 on pages 18 and 19). It is stated on page 19, lines 25-26 that "[T]he experiments

set forth above confirm that the relative level of MUC18 expression in prostate cancer cells correlates positively with metastatic ability".

The above studies led the inventor to predict that absence or very low MUC18 expression in the prostate cancer is associated with non-metastatic cancer and relatively high levels of MUC18 expression are predictive of prostate cancer which is likely to metastasize or which has already metastasized (*See* Abstract).

The predictions made in the disclosure were proven to be correct in later studies performed by the inventor using clinical samples of varying degree of pathologic stages of prostate cancer, from normal to metastatic carcinoma. The results confirmed the correlation between the expression of MUC18 and the progression of the prostate cancer. A Declaration under 37 C.F.R. § 1.132 by the inventor summarizing these studies was submitted with the Amendment filed on June 7, 2002. As shown in Table 1 on page 2, none of the BPH (benign prostrate hyperplasia) showed detectable MUC18 expression whereas progressively higher percentage (58% to 80%) of the subject from PIN to metastatic carcinoma showed a significant level of MUC18 expression (*See* column, "number of cases positive for huMUC18 (+2)" where 18 out of 31 of the PIN cases showed +2 levels of expression and 4 out of 5 of metastatic carcinoma showed +2 levels of expression. Please note that the +1 indicates a weak staining that is slightly above the background, as indicated in line 1 on page 3).

Based on the above, the claimed invention, i.e., a method of predicting a risk for metastasis of a prostate cancer cell based on the relative level of expression of MUC18, was developed. Applicant respectfully submits that the Specification does provide sufficient description to convey to one skilled in the art that the inventor had possession of the claimed invention. Withdrawal of the rejection under 35 U.S.C. § 112 is respectfully requested.

Conclusion:

Based on the foregoing amendments and arguments, it is submitted that this case is in condition for allowance and passage to issuance is respectfully requested.

If there are any outstanding issues related to patentability, the courtesy of a telephone interview is requested, and the Examiner is invited to call to arrange a mutually convenient time.

This Amendment is accompanied by a Petition for Extension of Time (three months) and a check in the amount of $475.00 as required under 37 C.F.R. 1.17(a)(3) for a small entity. However, if the amount submitted is incorrect, please charge any deficiency or credit any overpayment to Deposit Account No. 07-1969.

Respectfully submitted,

Heeja Yoo-Warren
Reg. No. 45,495

GREENLEE, WINNER AND SULLIVAN, P.C.
5370 Manhattan Circle, Suite 201
Boulder, CO 80303
Telephone: (303) 499-8080
Facsimile: (303) 499-8089
E-mail: winner@greenwin.com
Attorney docket No. 95-97
nk: 10/07/03

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 20, Issue of May 17, pp. 18182–18190, 2002
Printed in U.S.A.

Biosynthesis of HNK-1 Glycans on *O*-Linked Oligosaccharides Attached to the Neural Cell Adhesion Molecule (NCAM)

THE REQUIREMENT FOR CORE 2 β1,6-*N*-ACETYLGLUCOSAMINYLTRANSFERASE AND THE MUSCLE-SPECIFIC DOMAIN IN NCAM*

Received for publication, February 8, 2002, and in revised form, March 8, 2002
Published, JBC Papers in Press, March 12, 2002, DOI 10.1074/jbc.M201312200

Edgar Ong‡, Misa Suzuki, Frederic Belot§, Jiunn-Chern Yeh¶, Isabelle Franceschini‖, Kiyohiko Angata, Ole Hindsgaul, and Minoru Fukuda**

From the Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, California 92037

The HNK-1 glycan, sulfo→3GlcAβ1→3Galβ1→4-GlcNAcβ1→R, is highly expressed in neuronal cells and apparently plays critical roles in neuronal cell migration and axonal extension. The HNK-1 glycan synthesis is initiated by the addition of β1,3-linked GlcA to *N*-acetyllactosamine followed by sulfation of the C-3 position of GlcA. The cDNAs encoding β1,3-glucuronyltransferase (GlcAT-P) and HNK-1 sulfotransferase (HNK-1ST) have been recently cloned. Among various adhesion molecules, the neural cell adhesion molecule (NCAM) was shown to contain HNK-1 glycan on *N*-glycans. In the present study, we first demonstrated that NCAM also bears HNK-1 glycan attached to *O*-glycans when NCAM contains the *O*-glycan attachment scaffold, muscle-specific domain, and is synthesized in the presence of core 2 β1,6-*N*-acetylglucosaminyltransferase, GlcAT-P, and HNK-1ST. Structural analysis of the HNK-1 glycan revealed that the HNK-1 glycan is attached on core 2 branched *O*-glycans, sulfo→3GlcAβ1→3Galβ1→4-GlcNAcβ1→6(Galβ1→3)GalNAc. Using synthetic oligosaccharides as acceptors, we found that GlcAT-P and HNK-1ST almost equally act on oligosaccharides, mimicking *N*- and *O*-glycans. By contrast, HNK-1 glycan was much more efficiently added to *N*-glycans than *O*-glycans when NCAM was used as an acceptor. These results are consistent with our results showing that HNK-1 glycan is minimally attached to *O*-glycans of NCAM in fetal brain, heart, and the myoblast cell line, C2C12. These results combined together indicate that HNK-1 glycan can be synthesized on core 2 branched *O*-glycans but that the HNK-1 glycan is preferentially added on *N*-glycans over *O*-glycans of NCAM, probably because *N*-glycans are extended further than *O*-glycans attached to NCAM containing the muscle-specific domain.

Neural cell adhesion molecules undergo critical post-translational modifications that modulate their affinity and in some cases alter their specificity for their cognate ligands (1, 2). In particular, many neural cell adhesion molecules are heavily glycosylated, and the extent and composition of attached carbohydrates modify their adhesive properties. Among those, polysialic acid and HNK-1 are particularly notable. Polysialic acid is mostly attached to NCAM,[1] and polysialylated NCAM is abundantly present in embryonic brain (1, 2). In the adult brain, polysialylated NCAM is restricted to certain tissues, such as the hippocampus and olfactory bulb, where neuronal regeneration persists. Polysialic acid is a linear homopolymer of α-2,8-linked sialic acid residues formed on α-2,3- or α-2,6-linked sialic acid in *N*-acetyllactosamine of *N*-glycans (3–6). Polysialic acid is attached to two *N*-glycosylation sites in the fifth immunoglobulin-like domain in NCAM and thought to attenuate the adhesive property of NCAM (7–9).

The HNK-1 carbohydrate epitope was originally defined by a monoclonal antibody raised against human natural killer (HNK) cells (10). In nervous tissues, the HNK-1 carbohydrate was first recognized as an autoantigen involved in peripheral demyelinative neuropathy (11, 12). The structural analysis of glycolipids reacting with these autoantibodies demonstrated that the HNK-1 epitope is sulfo→3GlcAβ1→3Galβ1→4GlcNAcβ1→R (11, 12). HNK-1 glycan is widely distributed in glycoproteins, glycolipids, and proteoglycans (2, 11–13). The expression of HNK-1 glycan is spatially and developmentally regulated and found on migrating neuronal crest cells, cerebellum, and myelinating Schwann cells in motor neurons but not on those in the sensory neurons (14–16). Although it has been reported that HNK-1 glycan is attached to the *N*-glycosylation site in the third immunoglobulin-like domain of NCAM, the recent studies showed that HNK-1 glycan is attached to the second, third, fifth, and sixth *N*-glycosylation sites (17).

HNK-1 apparently plays critical roles in neural cell migration and axonal extension. HNK-1 glycolipids coated on plates facilitated neurite outgrowth, whereas no facilitation of neurite outgrowth was observed on sulfatide, 3′-sulfogalactosyl ceramide. The effect of HNK-1 glycan was abolished once the sulfate group was removed (18, 19). The HNK-1 glycan was identified in NCAM and *N*-glycans isolated from P0 glycoprotein (20, 21). It was also found as rather unusual structures of *O*-linked oligosaccharides, sulfo→3GlcAβ1→3Galβ1→4GlcNAcβ1→2Man, which then are attached to serine or threonine (22). This glycan can be

* This work was supported by NCI, National Institutes of Health, Grants R37CA33000, RO1CA33895, and PO1CA71932. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Corvas International Inc., San Diego, CA 92121.
§ Present address: Unite de Chimie Organique, Institute Pasteur, 75724 Paris cedex 15, France.
¶ Present address: Genset Corp., San Diego, CA 92121.
‖ Present address: Deptartment of Neuroscience, Institute Pasteur, 75724 Paris cedex 15, France.
** To whom correspondence should be addressed: The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-646-3144; Fax: 858-646-3193; E-mail: minoru@burnham.org.

[1] The abbreviations used are: NCAM, neural cell adhesion molecule; GlcA, glucuronic acid; MSD, muscle-specific domain; HNK-1ST, HNK-1 sulfotransferase; GlcAT-P, β1,3-glucuronyltransferase; VASE, variable alternatively spliced exon; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; GnT-I, β1,2-*N*-acetylglucosaminyltransferase I; Core2GlcNAcT, core 2 β1,6-*N*-acetylglucosaminyltransferase; ConA, concanavalin A.

attached to dystroglycan, and its defect may cause muscular dystrophy (23).

NCAM has numerous isoforms due to alternative splicing of precursor mRNAs. Among them, NCAM(MSD) contains an additional domain, the so-called muscle-specific domain (MSD), consisting of 37 amino acids between two fibronectin type III-like domains (24). This NCAM(MSD) is mostly present in skeletal myotubes and often anchored to the plasma membrane through glycophosphatidylinositol (24). The MSD is highly enriched with serine, threonine and proline and *O*-linked oligosaccharides were shown to attach to this domain (25). It has been shown that NCAM(MSD) from C2C12 myoblast cell line contains mucin-type *O*-linked oligosaccharides ±NeuNAcα2→3Galβ1→3(±NeuNAcα2→6)GalNAcα→Thr/Ser (25). However, no studies have addressed whether *O*-glycans in NCAM contain an HNK-1-capping structure.

The HNK-1 glycan is synthesized in a stepwise manner by the addition of a β1,3-linked glucuronic acid to precursor *N*-acetyllactosamine by β1,3-glucosaminyltransferase (GlcAT-P) followed by the addition of a sulfate group by HNK-1 sulfotransferase (HNK-1ST) to GlcAβ1→3Galβ1→4GlcNAc→R, forming sulfo→3GlcAβ1→3Galβ1→4GlcNAc→R (26). Recently, the cDNAs encoding GlcAT-P and HNK-1ST have been cloned (27–29). In mucin-type *O*-glycans, *N*-acetyllactosamine can be formed when core 2 branch, GlcNAcβ1→6(Galβ1→3)GalNAcα1→R, is synthesized by core 2 β1,6-*N*-acetylglucosaminyltransferase (Core2GlcNAcT, Fig. 1, Ref. 30). These results prompted us to examine how the HNK-1 glycan is synthesized in mucin-type *O*-linked oligosaccharides attached to NCAM.

In this report, we first present evidence that HNK-1 glycan can be formed in *O*-glycans attached to the MSD in NCAM. We found that HNK-1 glycan is attached to core 2 branched *O*-glycans but not to core 1 *O*-glycans present in NCAM. We determined the HNK-1 glycan structure in mucin-type *O*-linked oligosaccharides as sulfo→3GlcAβ1→3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAc. Finally, we found that HNK-1 glycans can be formed on *N*-glycans more efficiently than *O*-glycans on NCAM, although GlcAT-P and HNK-1ST utilize *O*-glycan and *N*-glycan oligosaccharides almost equally as an acceptor.

EXPERIMENTAL PROCEDURES

Plasmids Encoding NCAM, NCAM(MSD), GlcAT-P, and HNK-1ST—pIG-NCAM-IgG and pIG-NCAM(MSD)-IgG encoding NCAM-IgG and NCAM(MSD)-IgG chimeric proteins, respectively, were constructed from pIG-NCAM(VASE, MSD)-IgG, originally provided by Dr. David Simmons at Oxford University (31), as described previously (32). All products derived from these different vectors were found to react with anti-NCAM antibody (Eric-1) by Western blotting, confirming the sequences and sizes of the products (32).

The cDNA encoding rat β1,3-glucuronyltransferase, GlcAT-P (27), was cloned as described previously using reverse transcription-PCR and subcloned into pcDNA3, resulting in pcDNA3(neo)-GlcAT-P (29). The cDNA encoding a catalytic domain of GlcAT-P was obtained by PCR using pcDNA3(neo)-GlcAT-P as a template. 5′- and 3′-primers for the PCR are 5′-CGGATCCCAGCCTCGCACCTCTGCTTGCT-3′ (the *Bam*HI site is underlined, and the rest is nucleotides 120–141 of GlcAT-P) and 5′-ACTCGAGTCAGATCTCCACCGAGGGGTC-3′ (the *Xho*I site is underlined, and the rest of the sequence is nucleotides 1044–1024 of GlcAT-P). After *Bam*HI and *Xho*I digestion, the cDNA fragment was ligated into the same sites of pcDNAI-A, which harbors a signal peptide and IgG-binding domain of protein A, resulting in pcDNAI-A-GlcAT-P. The cDNA encoding a human sulfotransferase that transfers a sulfate group from PAPS to glucuronylated *N*-acetyllactosamine precursors was cloned into pcDNA3 as described previously, resulting in pcDNA3-HNK-1ST (29). Similarly, pcDNAI-A-HNK-1ST was prepared as described before (33). pcDNAI encoding core 2 β1,6-*N*-acetylglucosaminyltransferase, Core2GlcNAcT-I (30), was cloned into the pcDNA3.1(Zeo), resulting pcDNA3.1(Zeo)-Core2GlcNAcT-I. A cDNA encoding β1,2-*N*-acetylglucosaminyltransferase I (GnT-I) cloned in pcDNA3.1 (34, 35) was a kind gift of Dr. Pamela Stanley.

Preparation of Lec1 Cells Expressing HNK-1 Precursors and HNK-1 Glycans—To avoid the formation of HNK-1 in *N*-glycans, the majority of the experiments were carried out using a mutant Chinese hamster ovary cell line, Lec1. Since Lec1 cells are deficient in GnT-I, all of the *N*-glycans synthesized in this cell line remain as high mannose oligosaccharides (36). Lec1 cells were found to lack core 2 β1,6-*N*-acetylglucosaminyltransferase as in other Chinese hamster ovary cells (30). Lec1 cells were stably transfected with pcDNA3.1(Zeo)-Core2GlcNAcT-I as described before (29). Lec1-core 2 cells were first selected in the presence of zeocin (Invitrogen) and then by immunofluorescent staining using tomato lectin, which reacts with *N*-acetyllactosamine and poly-*N*-acetyllactosamines (37). As shown previously, the formation of core 2 branches results in the formation of small amounts of poly-*N*-acetyllactosamine (38). To confirm the integration of human Core2GlcNAcT-I cDNA into Lec1 cell chromosome, reverse transcription-PCR was carried out on poly(A)$^+$ RNA derived from Lec1-core 2 cells. The primers for this reverse transcription-PCR were designed in exon 1 and exon 2, avoiding the amplification of hamster genomic DNA harboring Core2GlcNAcT.

Lec1-core 2 cells were transfected with pcDNA3(neo)-GlcAT-P and selected in the presence of G418 and zeocin, and Lec1-core2-GlcA were chosen after immunostaining using M6749 antibody (39), which reacts with both nonsulfated and sulfated forms of HNK-1 carbohydrates (27, 29). These cells were further transfected with pcDNA3.1(hyg)-HNK-1ST and selected in the presence of G418, zeocin, and hygromycin. Those cells expressing a significant amount of HNK-1 glycan, assessed by immunostaining with anti-HNK-1 antibody (Becton Dickinson), were chosen and designated as Lec1-core 2-HNK-1. As the second antibody, fluorescein isothiocyanate-conjugated goat $(Fab')_2$ fragment specific to mouse IgG (for anti-NCAM antibody) or IgM (for HNK-1 antibody and M6749 antibody) was used. In parallel, Lec1 cells stably expressing GlcAT-P or HNK-1ST alone or GlcAT-P and HNK-1ST together were established.

Transient Expression of NCAM and NCAM(MSD)—Lec1 cells expressing the HNK-1 glycan were transiently transfected with pIG-NCAM-IgG or pIG-NCAM(MSD)-IgG with or without pcDNAI-Core2GlcNAcT-I, using LipofectAMINE Plus™ as described previously (29). The medium was changed to serum-free medium 24 h after the transfection and cultured for an additional 48 h. NCAM-IgG in the cultured medium was adsorbed to protein A-agarose as described before (8). NCAM-IgG molecules eluted from the medium were subjected to SDS-polyacrylamide (5%) gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blot was then incubated with anti-NCAM antibody, anti-HNK-1 antibody, or M6749 antibody, with a secondary antibody as described before (29).

Preparation of Lec2 Cells Expressing HNK-1 Precursor and HNK-1 Glycans—Lec2 cells expressing GlcAT-P and HNK-1ST, Lec2-HNK-1, were established as described previously (29). Lec2-HNK-1 cells were selected using anti-HNK-1 monoclonal antibody. Lec2 cells expressing GlcAT-P were similarly established and selected by immunofluorescent staining using M6749 monoclonal antibody. Lec2 cells do not synthesize sialylated oligosaccharides due to the defect in CMP-sialic acid transporter in the Golgi (40).

Metabolic Labeling of Carbohydrate Moiety of NCAM-IgG—Lec1-core 2-HNK-1 cells were transiently transfected with pIG-NCAM(MSD)-IgG or pIG-NCAM-IgG. Twenty-four hours after the transfection, the medium was replaced with sulfate-free or glucose-free RPMI 1640 medium containing dialyzed 10% fetal calf serum in the presence of $Na_2[^{35}S]O_4$ (100 μCi/ml), [6-^{3}H]$GlcNH_2$ (10 μCi/ml), and [6-^{3}H]Gal (20 μCi/ml) or [6-^{3}H]$GlcNH_2$ (10 μCi/ml) or $Na_2[^{35}S]O_4$ (100 μCi/ml) alone (41). After a 48-h incubation, the spent medium was harvested, and NCAM-IgG was isolated as described above. After confirming the purity of the isolated samples by SDS-polyacrylamide gel electrophoresis and fluorography, the samples were subjected to oligosaccharide characterization.

Isolation of O-Linked Oligosaccharides Containing HNK-1 Glycans—Metabolically labeled NCAM(MSD)-IgG was digested with Pronase, and labeled glycoproteins were isolated by Sephadex G-25 gel filtration as described previously (41). *O*-Glycan-containing glycopeptides were separated from high mannose glycopeptides and other glycans using ConA-Sepharose affinity chromatography. The purified glycopeptides were subjected to alkaline reductive treatment to release *O*-linked oligosaccharides as described previously (41). Oligosaccharides were applied to a Poly-Prep column (Bio-Rad) containing 2 ml of anion-exchange QAE-Sephadex A-25. The resin was equilibrated with 2 mM pyridine-acetate buffer, pH 5.5, and eluted in a stepwise manner with 6 ml each of 70 mM, 250 mM, and 1 M NaCl in 2 mM pyridine-acetate buffer, pH 5.5. Fractions (1 ml) were collected, and aliquots were taken for determination of radioactivity.

The oligosaccharides eluted with 1 M NaCl were digested with *Arthrobacter* sialidase (Sigma) to remove sialic acid and subjected to QAE-Sephadex A-25 column chromatography. Those eluted with 1 M NaCl after sialidase treatment were subjected to structural characterization.

Structural Characterization of O-Glycans Containing HNK-1 Glycan—The *O*-linked glycans were subjected to solvolysis to remove a sulfate group as described previously (41, 42). The resultant oligosaccharides, purified by QAE-Sephadex chromatography, were digested with 125 milliunits of *Escherichia coli* β-glucuronidase (Sigma) in 50 μl of the reaction mixture under the same conditions as described previously (11). The obtained oligosaccharides were analyzed on an AX-5 amino-bonded column using a Gilson 306 HPLC apparatus at a flow rate of 0.8 ml/min. The column was equilibrated with 90% solvent A (80% acetonitrile, 20% 15 mM KH_2PO_4) and 10% of solvent B (40% acetonitrile, 60% 15 mM KH_2PO_4). After 5 min, the elution was carried out by linear gradient from 10% of solvent B to 100% of solvent B in 60 min. The column was finally washed for 5 min with solvent B. Fractions were collected every minute, and aliquots were taken for determination of the radioactivity by scintillation counting.

Periodate Oxidation of Oligosaccharides—Oligosaccharides were oxidized with 50 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, at room temperature, and oxidized samples were reduced as described previously (43). The samples, after destroying $NaBH_4$ by acetic acid, were passed over a small column of Dowex 50 × 8 and dried after the addition of methanol. The samples were then hydrolyzed in 0.01 M HCl at 80 °C for 1 h, neutralized with 0.1 M Tris-HCl buffer, pH 8.5, and applied to a column (1 × 120 cm) of Bio-Gel P-4 (200–400-mesh) equilibrated with 0.1 M pyridine-acetate buffer, pH 5.5. Oligosaccharides were also separated by QAE-Sephadex A-25 column chromatography as described above.

Assays for GlcAT-P and HNK-1ST Activity—To assay the activities of GlcAT-P and HNK-1ST, soluble protein A-GlcAT-P and soluble protein A-HNK-1ST were prepared and adsorbed to IgG-Sepharose as described previously (33). To assay for GlcAT-P activity, the reaction mixture (100 μl) consisted of 500 μM acceptors, 0.1 M HEPES buffer, pH 6.5, 10 mM $MnCl_2$, 10 mM of galactonic acid-γ-lactone, 50 μl of the soluble chimeric enzyme attached to beads, and 50 μM UDP-[^{14}C]GlcA (294 mCi/mmol, PerkinElmer Life Sciences). The reaction mixture was incubated for 3 h at 37 °C, and the reaction was stopped by adding 50 mM (final concentration) EDTA.

To assay for HNK-1ST activity, the donor, 3′-phosphate 5′-phospho-[^{35}S]sulfate, and various amounts of an acceptor were incubated with a solution of the 50% suspension of the chimeric protein A-HNK-1ST attached to beads under the conditions described previously (29, 33). The reaction was stopped by 250 mM ammonium formate, pH 4, and labeled products were obtained by C18 reverse-phase chromatography as described previously (29). The K_m for GlcAT-P and HNK-1ST was determined using a Lineweaver-Burk plot at various concentrations of the acceptor (2.5–2000 μM).

Synthesis of Standard Oligosaccharides—The acceptors GlcAβ-1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→O$(CH_2)_7CH_3$ (octyl) (compound 1), GlcAβ1→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→O-$(CH_2)_7CH_3$ (octyl) (compound 2), and GlcAβ1→3Galβ1→4GlcNAcβ-1→6(Galβ1→3)GalNAcα1→O$(CH_2)_7CH_3$ (octyl) (compound 3) were synthesized from precursors octyl 3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl(1→6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl(1→6)-2,3,4-tri-*O*-benzyl-β-D-mannopyranoside (compound 4), octyl 3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl(1→2)-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl(1→6)-2,3,4-tri-*O*-benzyl-β-D-mannopyranoside (compound 5), and octyl 3,6-di-*O*-benzyl-2-deoxy-2-trichloroacetamido-β-D-glucopyranosyl(1→6)[2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl(1→3)]-2-acetamido-4-*O*-acetyl-2-deoxy-α-D-galactopyranoside (compound 6). Each acceptor (compounds 4–6) was glycosylated with the donor *O*-(methyl 2,3,4-tri-O-benzoyl-β-D-glucopyranosuluronate)-(1→3)-2,4,6-tri-O-benzoyl-α-D-galactopyranosyl trichloroacetimidate (compound 7) with catalytic trimethylsilyl trifluoromethanesulfonate to give the derivatives of the pentasaccharides in good to excellent yield. Reduction of the trichloroacetamydoyl group to the acetimidoyl group (44) followed by the usual deprotection steps provided the target compounds 1–3.

The products were characterized by ^{1}H and ^{13}C NMR spectroscopy and high resolution electrospray-time-of-flight mass spectrometry.

Partial ^{1}H NMR (600 MHz, D_2O) (compounds 1 and 2); (300 MHz, D_2O) (compound 3); are as follows. Compound 1: δ 4.894 (d, $J_{1,2}$ = 1.5 Hz, α-Man H-1), 4.678 (d, $J_{1,2}$ = 7.8 Hz, GlcA H-1), 4.666 (s, $J_{1,2}$ < 1.0 Hz, β-Man H-1), 4.586 (d, $J_{1,2}$ = 8.3 Hz, GlcNAc H-1), 4.533 (d, $J_{1,2}$ = 7.9 Hz, Gal H-1), 2.0 (s, 3 H, NHAc). Compound 2: δ 4.930 (d, $J_{1,2}$ = 1.6 Hz, α-Man H-1), 4.678 (d, $J_{1,2}$ = 7.8 Hz, GlcA H-1), 4.666 (s, $J_{1,2}$ < 1.0 Hz, β-Man H-1), 4.610 (d, $J_{1,2}$ = 7.8 Hz, GlcNAc H-1), 4.528 (d, $J_{1,2}$ = 7.9 Hz, Gal H-1), 2.0 (s, 3 H, NHAc). Compound 3: δ 4.85 (d, $J_{1,2}$ = 3.5 Hz, GalNAc H-1), 4.65 (d, $J_{1,2}$ = 7.7 Hz, GlcA H-1), 4.50, 4.49, and 4.48 (3 d, $J_{1,2}$ = 8.2 Hz, $J_{1,2}$ = 8.5 Hz, $J_{1,2}$ = 7.6 Hz, 2 Gal H-1 and GlcNAc H-1), 1.98 and 1.96 (2s, 6 H, NHAc). *m/z* calc. for $C_{40}H_{69}N_1Na_1O_{27}$ (compounds 1 and 2): 1018.3955; found compound 1: 1018.3961; compound 2: 1018.3959. Detailed procedures of the synthesis will be published elsewhere.[2] Galβ1→4GlcNAcβ1→octyl, Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl, Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl, and Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα1→octyl were synthesized as described previously (45–47).

RESULTS

Expression of HNK-1 Glycan in Mucin-type O-Glycans Attached to NCAM—To determine the biosynthetic pathway of HNK-1 glycan in mucin-type oligosaccharides attached to NCAM, Chinese hamster ovary mutant Lec1 cells were utilized. Lec1 cells contain the deficient *N*-acetylglucosaminyltransferase I, which is the first key enzyme to form complex *N*-glycans (36). Because this enzyme is absent in Lec1 cells, *N*-glycans remain as the high mannose type, and *N*-acetyllactosamine, which is a precursor for HNK-1 glycan, is not formed in *N*-glycans.

Lec1 cells also lack core 2 β-1,6-*N*-acetylglucosaminyltransferase (Core2GlcNAcT) as in wild-type Chinese hamster ovary cells. Since mucin-type oligosaccharides can acquire *N*-acetyllactosamine when core 2 branches are formed by Core2GlcNAcT, Lec1 cells expressing HNK-1 glycan (Lec1-HNK-1) were further transfected with cDNA encoding Core2GlcNAcT-I (see Fig. 1). As shown in Fig. 2*A*, HNK-1 was detected by Western blotting in NCAM(MSD)-IgG synthesized in Lec1 cells expressing HNK-1ST and GlcAT-P, together with Core2GlcNAcT-I (*lanes 4*). When HNK-1ST was absent, glucuronylated precursor glycan was detected by anti-glucuronic acid antibody, M6749 (GlcA), but not by anti-HNK-1 antibody (HNK-1) (*lanes 6* in Fig. 2*A*). Multiple HNK-1-positive bands may represent NCAM(MSD)-IgG containing different numbers of sulfate groups, since glucuronylated molecules correspond mainly to the lowest molecular weight band (Fig. 2*A*, *lane 6*). Moreover, NCAM(MSD)-IgG did not react with either anti-HNK-1 antibody or M6749 antibody when Lec1 cells were not transfected with Core2GlcNAcT cDNA (Fig. 2*A*, *lanes 1–3*). These results establish that Core2GlcNAcT, GlcAT-P, and HNK-1ST are all necessary to form the HNK-1 glycan in NCAM(MSD).

To determine whether HNK-1 glycans can be attached to *O*-glycans in sequences other than MSD, NCAM-IgG lacking the MSD sequence was also expressed in the same Lec1 cells expressing Core2GlcNAcT-I and HNK-1ST glycan on the cell surface. As shown in Fig. 2*B*, NCAM synthesized in these Lec1 cells did not bear the HNK-1 glycan or glucuronylated precursor regardless of whether Core2GlcNAcT was present or absent. Minor HNK-1-positive glycoproteins were detected when Lec1 cells expressed Core2GlcNAcT-I, GlcAT-P, and HNK-1ST (Fig. 2*B*, *lane 4*). They were judged to be contaminating glycoproteins other than NCAM based on their mobility in SDS-polyacrylamide gel electrophoresis. These results indicate that the MSD sequence is essential for acquiring the HNK-1 glycan in *O*-glycans attached to NCAM.

Structural Characterization of the HNK-1 Glycan Attached to the MSD Sequence in NCAM—To determine the structure of the HNK-1 glycan attached to NCAM, NCAM(MSD)-IgG was transiently expressed in Lec1-core 2-HNK-1 cells expressing HNK-1 glycan and core 2 branched *O*-glycans. Twenty-four h

[2] F. Belot, M. Fukuda, and O. Hindsgaul, manuscript in preparation.

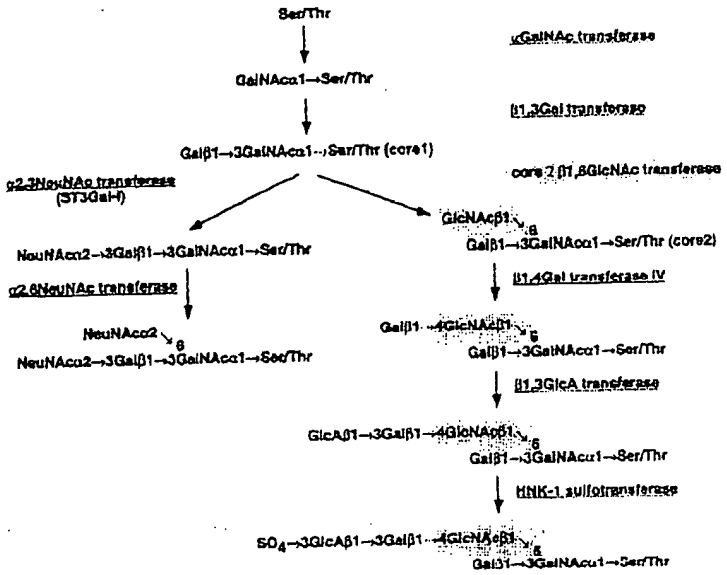

FIG. 1. **Biosynthetic pathway of HNK-1 glycan on mucin-type *O*-glycans.** Core 1 *O*-glycans can be converted to core 2 *O*-glycans, followed by galactosylation by β4GalT-IV. The resultant *N*-acetyllactosaminyl core 2 *O*-glycans can be converted by β1,3-glucuronyltransferase (*β1,3-GlcAT*) and HNK-1 sulfotransferase (*HNK-1ST*) to HNK-1 glycan on core 2 branched *O*-glycans (*right*). On the other hand, core 1 *O*-glycans can be sialylated by ST3Gal-I followed by α2,6-sialyltransferases, which form disialyl core 1 *O*-glycans (*left*), based on the present and previous studies (59–61).

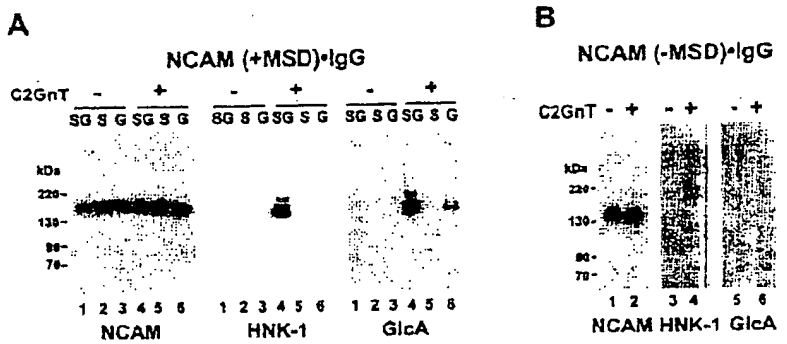

FIG. 2. **Western blot analysis of NCAM-IgG chimeric protein in Lec1 cells transfected with GlcAT-P and HNK-1ST.** *A*, Lec1 cells expressing HNK-1ST (*S*), GlcAT-P (*G*), or both HNK-1ST and GlcAT-P (*SG*) were transiently transfected with NCAM(MSD)-IgG chimeric protein together with (+) or without (−) Core2GlcNAcT-I (*C2GnT*). Released chimeric NCAM-IgG was purified by protein A column and subjected to Western blot analysis after separating SDS-polyacrylamide gel electrophoresis. The blot was reacted with anti-NCAM (*N-CAM*), anti-HNK-1 (*HNK-1*), or anti-glucuronic acid (*GlcA*) M6749 antibody. *B*, Lec1 cells expressing both HNK-1ST and GlcAT-P were transiently transfected with NCAM-IgG (devoid of MSD) together with (+) or without (−) Core2GlcNAcT-I (*C2GnT*). NCAM-IgG was analyzed in the same manner as in *A*.

after the transfection, the transfected cells were cultured for an additional 48 h in sulfate-free medium in the presence of [^{3}H]GlcN, [^{3}H]Gal, and [^{35}S]sulfate. Similarly, the transfected cells were cultured in glucose-free medium with [^{3}H]GlcN or in sulfate-free medium with [^{35}S]sulfate. NCAM(MSD)-IgG molecules purified from the cultured medium were then subjected to SDS-polyacrylamide gel electrophoresis and fluorography. The results showed that [^{35}S]sulfate, [^{3}H]galactose, and [^{3}H]glucosamine were incorporated into NCAM(MSD)-IgG (data not shown).

As a first series of experiments for structural characterization, NCAM(MSD)-IgG labeled with [^{3}H]galactose, [^{3}H]glucosamine, and [^{35}S]sulfate was used. Glycopeptides were prepared and applied to ConA-Sepharose chromatography. The results shown in Fig. 3 demonstrated that ^{35}S-labeled material was not bound to ConA-Sepharose. The glycopeptides eluted with 10 mM methyl α-glucoside and 250 mM methyl α-mannoside represent biantennary and high mannose type *N*-glycans, respectively, and they did not contain sulfate. The glycopeptides unbound to ConA-Sepharose (shown in the *horizontal bar* in Fig. 3), representing *O*-glycans and multiantennary *N*-glycans, were then subjected to alkaline borohydride treatment to release *O*-linked oligosaccharides.

The treated sample was separated into two peaks after Sephadex G-25 gel filtration. The first peak of higher molecular weight contained both ^{35}S and ^{3}H radioactivity, whereas the second peak contained only ^{3}H radioactivity (data not shown). Upon QAE-Sephadex column chromatography, all of the ^{35}S-labeled material was recovered in the fraction eluted with 1 M NaCl in the pyridine-acetate buffer (Fig. 4*A*, Q3). This ^{35}S- and ^{3}H-labeled material eluted again in this highly anionic fraction after sialidase treatment (Fig. 4*B*). Since no ^{3}H radioactivity was released, peak Q3 did not contain sialic acid, which can be labeled from [^{3}H]glucosamine precursor. In contrast, the glycopeptides eluted with 2–250 mM NaCl (Q0, Q1, and Q2 in Fig. 4*A*) were mostly eluted at the void volume after sialidase treatment (data not shown), consistent with the fact that they were devoid of [^{35}S]sulfate.

Since no sulfatase isolated to date is known to remove a sulfate group from HNK-1 glycan, we opted to remove sulfate by solvolysis. The sample after solvolysis eluted with 70 mM NaCl (Fig. 4*C*, *peak b*), where material containing one acidic group elutes. There was still substantial radioactivity eluted with 250 mM and 1 M NaCl (Fig. 4*C*, *peak c*). The latter material was mostly free [^{35}S]sulfate (fractions 24–32 in Fig. 4*D*), while a small amount of remaining HNK-1 glycan existed (*peak d* in Fig. 4*D*). *Peak b* in Fig. 4*C*, desulfated HNK-1 glycan, was subjected to Bio-Gel P-4 gel filtration before and after β-glucu-

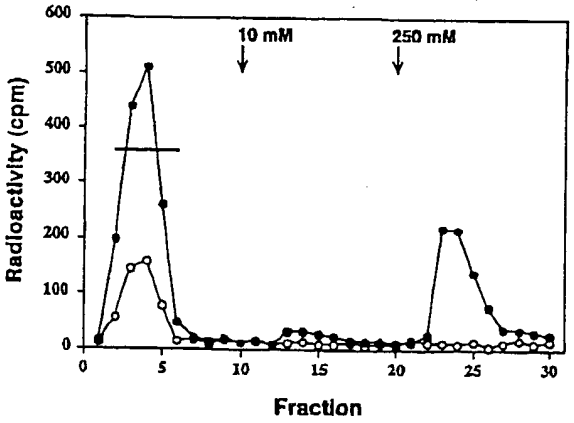

FIG. 3. **Separation of glycopeptides derived from metabolically labeled NCAM(MSD)-IgG by concanavalin A-Sepharose column.** Lec1-core2-HNK-1 cells were transiently transfected with NCAM(MSD)-IgG in the presence of [^{35}S]sulfate, [^{3}H]GlcN, and [^{3}H]Gal, and NCAM(MSD)-IgG was isolated from the cultured medium. Glycopeptides prepared from radiolabeled NCAM(MSD)-IgG were applied to a column of ConA-Sepharose. After washing the column, it was eluted with 10 mM methyl α-glucoside and 250 mM methyl α-mannoside. *Open* and *closed circles* represent ^{35}S and ^{3}H radioactivity, respectively.

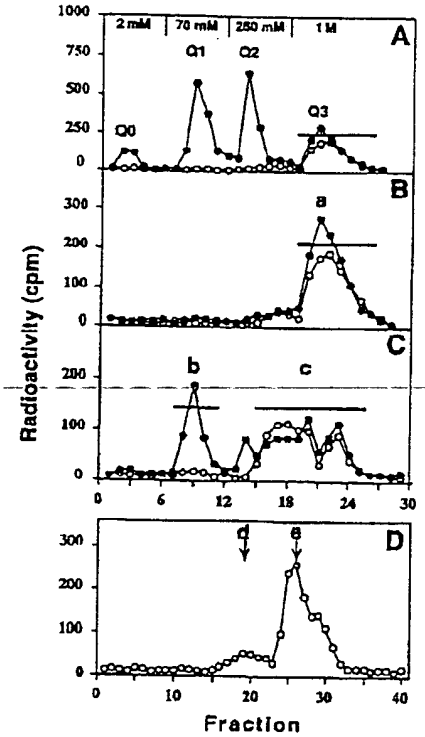

FIG. 4. **QAE-Sephadex column chromatography and Bio-Gel P-4 gel filtration of released *O*-glycans.** *A*, *O*-glycans were prepared from glycopeptides designated by a *horizontal bar* in Fig. 3 and separated by QAE-Sephadex column chromatography. *B* and *C*, *O*-glycans eluted by 1 M NaCl (shown by a *horizontal bar* in *A*) were subjected to the same QAE-Sephadex column chromatography after sialidase treatment (*B*) or removal of sulfate by solvolysis (*C*). *D*, *peak c* in *panel C* was applied for a short column of Bio-Gel P-4. *Peaks d* and *e* denote the elution positions of intact sulfated HNK-1 glycan and free sulfate, respectively. *Open* and *closed circles* represent ^{35}S and ^{3}H radioactivity, respectively.

ronidase treatment. The isolated oligosaccharide, *peak b* in Fig. 5*B*, was converted by β-glucuronidase digestion to Galβ1→4GlcNAcβ1→6(Galβ1→4)GalNAcOH, *peak c* in Fig. 5*C*. These results established that HNK-1 glycan, shown as *peak a* in Fig. 4*B*, has the structure sulfate→GlcAβ1→Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH.

Periodate Oxidation of HNK-1 Glycan and Its Desulfated Form—The above results establish that the HNK-1 glycan is attached to core 2 branched *O*-glycans. The above experiments, however, did not determine the linkage and attachment sites of sulfate and glucuronic acid. In order to obtain this information, the HNK-1 glycan and its nonsulfated form (*peaks a* and *b* in Fig. 5, *A* and *B*, respectively) were subjected to periodate oxidation followed by reduction and mild acid hydrolysis (Smith degradation). Periodate oxidation takes place in a *cis*-glycol group.

As a control experiment, core 2 *O*-glycan, Galβ1→4GlcNAcβ-1→6(Galβ1→3)GalNAc (Fig. 5*C*, *c*) was subjected to Smith degradation. This treatment produced two peaks: *f* and *g* (Fig. 5*F*). *Peaks f* and *g* represent GlcNAc and glycerol derived from galactose, respectively. *Peak c* derived from HNK-1 glycans yielded the same results. When *peak b* in Fig. 5*B*, nonsulfated HNK-1 glycan, was subjected to the same treatment, *peaks e* and *g* were produced (Fig. 5*E*). *Peak e* corresponds to Galβ1→4GlcNAc, which was confirmed by exoglycosidase digestion. These results show that glucuronic acid prevented galactose from oxidation, indicating that glucuronic acid residue was attached to C-3 of galactose. Otherwise, *peak b* in Fig. 5*B* and *peak c* in Fig. 5*C* would produce the same product. When *peak a* in Fig. 5*A* was subjected to the same treatment, *peak d* was obtained (Fig. 5*D*). *Peak d* was larger than *peak e* and was judged to be produced from sulfated glycans. *Peak e* in Fig. 5*D* was probably produced from nonsulfated glycans that were formed during various preparation steps. These results thus indicate that sulfate protected the GlcA residue from oxidation, and *peak d* corresponds to sulfo→3GlcAβ1→3Galβ1→4GlcNAc. The presence of sulfate and glucuronic acid in *peak d* was confirmed by the fact that *peak d* still had two anionic charges as assessed by QAE-Sephadex column chromatography (data not shown).

These results, combined together, establish the structure of HNK-1 glycan as sulfo→3GlcAβ1→3Galβ1→4GlcAcβ1→6(Galβ1→3)GalNAc.

NCAM in Mouse Embryonic Brain and Heart and C2C12 Cells Contain Negligible Amounts of HNK-1 Glycans Attached to O-Glycans—We then examined whether NCAM in mouse embryonic brain and heart contain HNK-1 glycans attached to *O*-glycans. NCAM present in brain at embryonic day 12 exhibit broad high molecular weights that were converted to bands of 200 and 140 kDa after *N*-glycanase treatment (Fig. 6*A*, *lanes 1* and *2*). When the same blot was incubated with anti-HNK-1 antibody, no band corresponding to *N*-glycanase-treated NCAM reacted with the antibody (Fig. 6*A*, *lanes 2* and *6*). The same experiment showed that small amounts of HNK-1 glycan were attached to *N*-glycans in NCAM, displaying two bands that are slightly larger than 220 kDa (Fig. 6*A*, *lane 5*). The remaining HNK-1 antigen was mostly associated with molecules larger than 220 kDa, suggesting that those HNK-1 glycans are associated with proteoglycans. HNK-1 was barely detected in heart tissue, although NCAM was detected (Fig. 6*A*, *lanes 3*, *4*, *7*, and *8*).

By using similar methods, the myoblast cell line, C2C12, was subjected to analysis. C2C12 cells were derived from mouse cells and can be induced to form myotubes (25). Our preliminary results showed that HNK-1 glycan was barely detected by Western analysis of cell lysates. We thus use ^{35}S-sulfate incorporation to detect sulfated glycans. The results shown in Fig. 6*B* illustrate that after 4 days of induced differentiation, C2C12 cells expressed [^{35}S]sulfate molecules that were heterogeneous

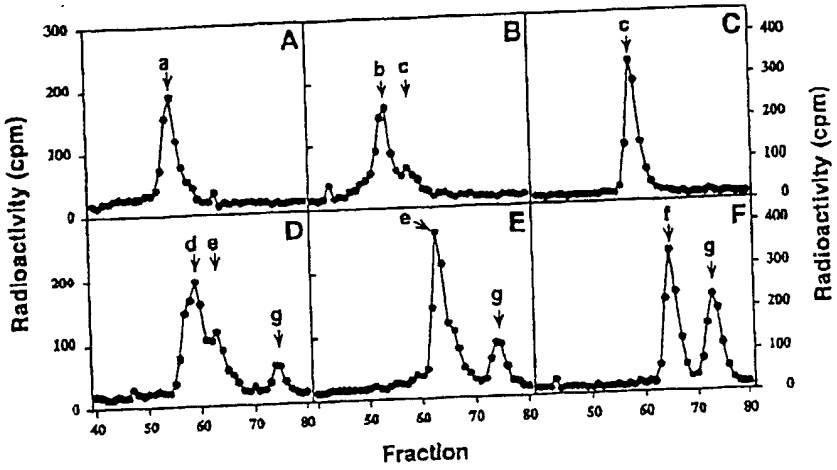

FIG. 5. **Bio-Gel P-4 gel filtration of HNK-1 glycans before and after various treatments.** *A–C*, *peak a* in Fig. 4*B* (*A*) and *peak b* in Fig. 4*C* before (*B*) and after β-glucuronidase treatment (*C*) were subjected to Bio-Gel P-4 gel filtration. *D–F*, the samples shown in *A–C* were subjected to Smith degradation and subjected to the same Bio-Gel P-4 gel filtration. *Peaks a–c* correspond to sulfo→3GlcAβ-1→3Galβ1→4GlcNAcβ1→6(Galβ1→3)-GalNAcOH, GlcAβ1→3Galβ1→4GlcNAc-β1→6(Galβ1→3)GalNAcOH, and Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH, respectively. *Peaks d–g* are explained under "Results." Galβ1→4GlcNAcβ1→6-(Galβ1→3)GalNAcOH was also prepared from HL-60 cells as described previously (43).

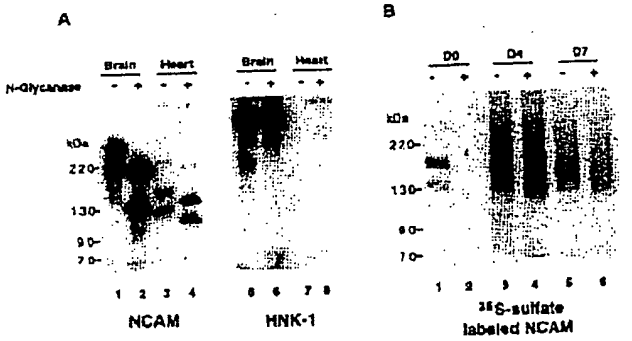

FIG. 6. **HNK-1 glycan present in mouse fetal brain and heart and C2C12 myoblast cells.** *A*, lysates derived from mouse brain and heart at embryonic day 12 were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western analysis using anti-NCAM (*lanes 1–4*) or anti-HNK-1 (*lanes 5–8*) antibody. The lysates were subjected to analysis before (−) and after (+) *N*-glycanase treatment. *B*, NCAM was immunoprecipitated from the cell lysates of [^{35}S]sulfate-labeled C2C12 cells and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

in molecular sizes. However, none of this polydispersed radioactive band was converted by *N*-glycanase treatment to a sharp band (Fig. 6*B*, *lanes 1–4*), whereas NCAM in day 0 and 4 culture apparently contained sulfate groups in *N*-glycans (Fig. 6*B*, *lanes 1–4*). It is possible that NCAM in days 4 and 7 may contain polysialic acid attached to *O*-glycans, or these broad bands may represent contaminating proteoglycans such as heparan sulfate. These results indicate that NCAM in C2C12 cells bear minimum amounts of HNK-1 glycan.

HNK-1 Glycan Synthesis Takes Place Preferentially on N-Glycans, although Both GlcAT-P and HNK-1ST Almost Equally Act on N- and O-Glycan Oligosaccharide Acceptors— The above results could be obtained if one of the glycosyltransferases responsible for HNK-1 glycans prefers *N*-glycans acceptors over *O*-glycan acceptors. To test this hypothesis, we first assayed β1,3-glucuronyltransferase (GlcAT-P) using various synthetic acceptors. The results shown in Fig. 7 illustrate that GlcAT-P utilizes synthetic acceptor oligosaccharides that mimic both *N*- and *O*-glycans. GlcAT-P utilizes Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl slightly better than core 2 branched *O*-glycan, but core 2 branched *O*-glycan acceptor is a slightly better acceptor than Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl. We then compared HNK-1ST activity in three different acceptors. As shown in Fig. 8, HNK-1ST acts slightly better on GlcAβ1→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl (K_m = 169 μM) than GlcAβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl (K_m = 358 μM). Core 2 branched *O*-glycan is an intermediate between these *N*-glycan acceptors (K_m = 283 μM). These results combined indicate that core 2 branched *O*-glycan is as good an acceptor as *N*-glycans for both GlcAT-P and HNK-1ST.

Despite the fact that the enzymes responsible for HNK-1 synthesis can utilize *N*-glycan and *O*-glycan acceptors almost equally well, HNK-1 was barely detected in NCAM *O*-glycans in tissues examined. These results obtained as a whole thus suggest that HNK-1 may be more efficiently added to *N*-glycans than *O*-glycans when they are attached to NCAM. In order to test this hypothesis, Lec1 cells expressing GlcAT-P and HNK-1ST were transiently transfected with cDNA encoding GnT-I. This transfection allows Lec1 cells to gain complex *N*-glycans, which can then become substrates for both GlcAT-P and HNK-1ST. The amount of HNK-1 glycan was significantly increased in NCAM(MSD) derived from Lec1 cells expressing GnT-I, compared with NCAM(MSD) derived from the parent Lec1 cells (Fig. 9*A*, compare *lanes 7* and *9*). The large majority of HNK-1 glycans were removed after *N*-glycanase treatment (Fig. 9*A*, *lane 10*), indicating that the majority of HNK-1 glycan is associated with newly synthesized *N*-glycans.

It is possible that sialylation inhibits HNK-1 glycan formation in core 2 branched *O*-glycans, since sialylation and glucuronylation compete for the same *N*-acetyllactosamine. To test this possibility, Lec2 cells that are deficient in sialylation were used to express HNK-1 glycan. Transfected Lec2 cells bearing both *N*-glycans and *O*-glycans expressed significant amounts of HNK-1 glycans, but almost all HNK-1 glycans were removed after *N*-glycanase treatment (Fig. 9*A*, *lanes 11* and *12*). Almost identical results were obtained when NCAM(MSD) were labeled with [^{35}S]sulfate (Fig. 9*B*). These results as a whole indicate that HNK-1 synthesis is shifted from *O*-glycans to *N*-glycans once *N*-glycan acceptor substrates are available.

DISCUSSION

The present study demonstrated that HNK-1 can be synthesized in *O*-glycans when three enzymes, GlcAT-P, HNK-1ST, and Core2GlcNAcT, are transfected into Lec1 cells, which lack the ability to form the HNK-1 glycan attached to complex and hybrid *N*-glycans. Since Lec1 cells lack Core2GlcNAcT, a simultaneous expression of Core2GlcNAcT is essential to form HNK-1 glycan on *O*-glycans. Our structural analysis of HNK-1 glycan indeed demonstrated that HNK-1 glycan is synthesized on *N*-acetyllactosamine in core 2 branched *O*-glycans (Fig. 1). This requirement of Core2GlcNAcT was also demonstrated for sialyl Lewis x synthesis in mucin-type *O*-glycans, since sialyl Lewis x, Siaα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R, is also

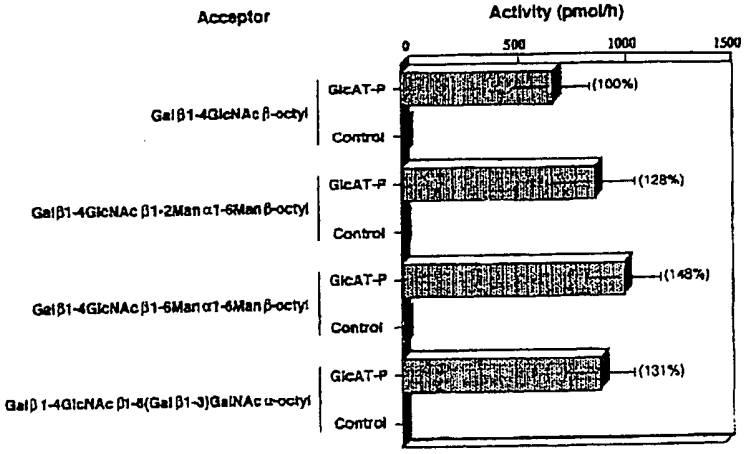

FIG. 7. **Incorporation of ^{14}C-GlcA by GlcAT-P to various acceptors.** A soluble chimeric GlcAT-P was incubated with UDP-[^{14}C]GlcA and acceptors shown. As a control, the culture medium derived from mock-transfected cells was used as an enzyme source.

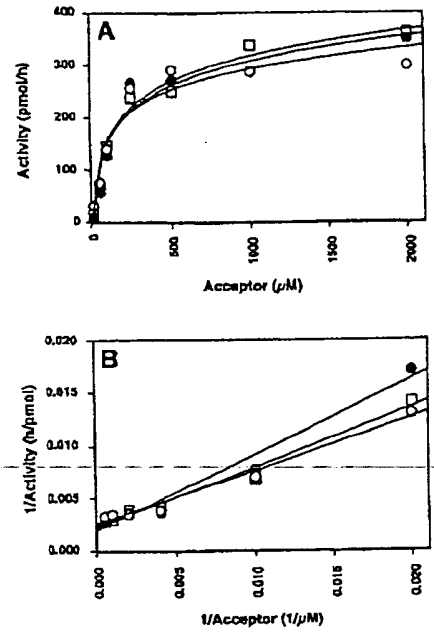

FIG. 8. **Dependence of HNK-1ST activity on the concentrations of various acceptors.** A soluble chimeric HNK-1ST was incubated with [^{35}S]PAPS and various concentrations of acceptors. *B*, the Lineweaver-Burk plot of *A*. The activity was for GlcAβ1→3Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl (○), GlcAβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl (●), and GlcAβ1→3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNACα1→octyl (□).

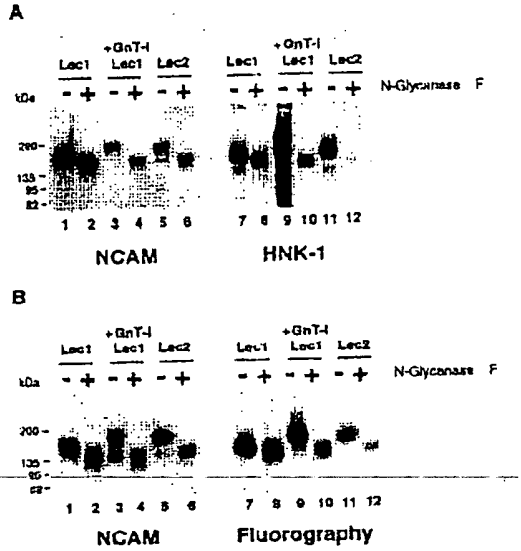

FIG. 9. **NCAM(MSD)-IgG released from Lec1 and Lec2 cells in the presence or absence of GnT-I.** Lec1 and Lec2 cells stably expressing HNK-1 glycan were transiently transfected with NCAM(MSD)-IgG in the absence (−) or presence (+) of GnT-I. *A*, NCAM(MSD)-IgG isolated was subjected to Western analysis before (−) or after (+) *N*-glycanase treatment. *B*, the transfected cells were metabolically labeled with [^{35}S]sulfate and released NCAM(MSD)-IgG was purified by protein A-conjugated resin. The *left panel* shows Western blot analysis using anti-NCAM antibodies to adjust the amount of NCAM(MSD)-IgG for assaying anti-HNK-1 reactivity (*A*) or fluorography (*B*). In *lane 9* of *panel A*, contaminating glycoproteins released from the Lec1 cells also contained HNK-1 glycan.

synthesized on *N*-acetyllactosamine backbone, which is formed by Core2GlcNAcT (41, 48).

The present study also demonstrated that mucin-type *O*-glycans bearing HNK-1 can be synthesized only when NCAM contains an MSD. MSD is enriched with threonine, serine, and proline, which is characteristic for amino acid sequences that attach mucin-type *O*-glycans (23, 24). A short amino acid sequence that consists of these amino acids is also present in the other part of NCAM, but it apparently does not serve for *O*-glycosylation attachment sites. MSD is encoded by four different exons, and differential splicing of NCAM precursor mRNA leads to the formation or absence of MSD. The expression of MSD-containing NCAM is temporally and spatially restricted, and myotubes express NCAM containing MSD (24).

Since the amino acid sequence of MSD is enriched with proline, threonine, and serine, MSD most likely does not have a unique conformation, such as an α-helical or β-sheet structure (49). Since *O*-glycosylation takes place in the Golgi apparatus, *O*-glycosylation sites need to be exposed to the environment after a protein folds. Non-α-helical and non-β-sheet structure satisfies such a requirement. In addition, it is most likely that such an amino acid sequence does not take a particular conformation, and its structure is flexible (49). It is thus possible that MSD may function as a hinge region where *O*-glycans are attached. This situation is similar to the hinge region of IgA, where multiple *O*-glycans are attached (50, 51). Lysosomal membrane glycoproteins (LAMP-1 and LAMP-2) also contain a hingelike structure, and the hingelike structure of human LAMP-1 has homology to the IgA hinge region (52, 53). Future studies are of importance to determine whether MSD in NCAM also functions as a hingelike structure.

Despite the fact that HNK-1 glycan can be added to *O*-glycans to NCAM(MSD) upon transfection with GlcAT-A and HNK-1, NCAM isolated from embryonic brain, heart, and C2C12 cells did not bear detectable amounts of HNK-1 epitope in *O*-glycans. This is most likely not due to the absence of Core2GlcNAcT, since Core2GlcNAcT-I is expressed in the brain and heart (54). One possible explanation for this discrepancy is that the expression levels of GlcAT-P and HNK-1ST are lower in these tissues compared with Lec1 cells or HeLa cells (data not shown) transfected with GlcAT-P and HNK-1ST. On the other hand, the same experiment clearly detected HNK-1 glycan in molecules with high molecular weights. Since those HNK-1-positive molecules still showed a polydispersity after *N*-glycanase treatment, it is likely that these molecules represent proteoglycans. These results indicate that the method used was sensitive enough to detect HNK-1 glycan. As a whole, it can be concluded that HNK-1 glycan is only barely, if at all, added on *O*-glycans attached to NCAM in the tissues examined.

The above results could be obtained if GlcAT-P and HNK-1 utilize *N*-glycans as acceptors better than *O*-glycans. However, GlcAT-P and HNK-1ST did not utilize *N*-glycan acceptors better than *O*-glycans when synthetic oligosaccharides that mimic a portion of *N*-glycans or *O*-glycans were used as acceptors. Moreover, the synthesis of HNK-1 glycans on NCAM was substantially increased when Lec1 cells were converted to synthesize complex-type *N*-glycan acceptors for HNK-1 glycan by transfecting with GnT-I. The amount of HNK-1 glycan on *O*-glycans is much less than that on *N*-glycans (for Lec1 cells bearing complex type *N*-glycans). These results indicate that GlcAT-P and HNK-1ST are more accessible to *N*-glycan acceptors than to *O*-glycan acceptors in glycoproteins such as NCAM. This is probably because *N*-glycans are more extended from the polypeptide backbone than *O*-glycans. On the other hand, PSGL-1 glycoprotein containing numerous *O*-glycans has an extended rodlike structure and has been shown to have sulfated *N*-acetyllactosamines, presumably on the *O*-glycans (55, 56). These combined results strongly suggest that MSD of NCAM may not display as preferable a conformation for enzymes that synthesize *O*-glycans as the conformation of PSGL-1.

HNK-1 glycan has been shown to bind to laminin (2), whereas sialylated core 1 *O*-glycan, NeuNAcα2→3Galβ1→3-(NeuNAcα2→6)GalNAc, was shown to bind Siglec such as myelin-associated glycoprotein (57). These results suggest that the addition of a HNK-1-capping structure on NCAM *O*-glycans may switch the interaction of NCAM with myelin-associated glycoprotein to that with laminin. Such switching probably plays a critical role in neural cell development.

Our studies demonstrated that HNK-1 synthesis may be synthesized in a complex manner and depends on how much *N*-glycan acceptors *versus* *O*-glycan acceptors are available. Similarly, it has been demonstrated that GlcNAc-6-*O*-sulfotransferase preferentially acts on *N*-glycans when glycoprotein acceptors such as CD34 bear both *N*- and *O*-glycans (41). By contrast, the same enzyme can act efficiently on *O*-glycans when the enzyme acts on GlyCAM-1, which almost exclusively contains *O*-glycans (58). In addition, it has been demonstrated that *N*-acetyllactosamine formation in core 2 branched *O*-glycans is much less efficient than in *N*-glycans (59). These results suggest that the synthesis of *N*-acetyllactosamine in core 2 glycans can be a rate-limiting step for the addition of HNK-1 epitope structure on *O*-glycans. These results combined indicate that the synthesis of terminal, functional oligosaccharide groups in *O*-glycans is regulated in a complex manner and that the availability of acceptors in *N*- or *O*-glycans and their conformation play a critical role in displaying such functional groups in different glycans.

Acknowledgments—We thank Dr. David Simmons for the pIG-NCAM (VASE, MSD), Drs. Pamela Stanley and Harry Schachter for the gift of the GnT-I cDNAs, Dr. Junya Mitoma for useful discussion, and Tracy Keeton for organizing the manuscript.

REFERENCES

1. Edelman, G. M., and Crossin, K. L. (1991) *Annu. Rev. Biochem.* 60, 155–190
2. Schachner, M., and Martini, R. (1995) *Trends Neurosci.* 18, 183–191
3. Finne, J. (1982) *J. Biol. Chem.* 257, 11966–11970
4. Kudo, M., Kitajima, K., Inoue, S., Shiokawa, K., Morris, R. R., Dell, A., and Inoue, Y. (1998) *J. Biol. Chem.* 271, 32687–32677
5. Angata, K., Suzuki, M., McAuliffe, J., Ding, Y., Hindsgaul, O., and Fukuda, M. (2000) *J. Biol. Chem.* 275, 18594–18601
6. Nakayama, J., Angata, K., Ong, E., Katsuyama, T., and Fukuda, M. (1998) *Pathol. Int.* 48, 665–677
7. Nelson, R. W., Bates, P. A., and Rutishauser, U. (1995) *J. Biol. Chem.* 270, 17171–17179
8. Angata, K., Suzuki, M., and Fukuda, M. (1998) *J. Biol. Chem.* 273, 28524–28532
9. von der Ohe, M., Wheeler, S. F., Wuhrer, M., Harvey, D. J., Liedtke, S., Mühlenhoff, M., Gerardy-Schahn, R., Geyer, H., Dwek, R. A., Geyer, R., Wing, D. R., and Schachner, M. (2002) *Glycobiology* 12, 47–63
10. Abo, T., and Balch, C. M. (1981) *J. Immunol.* 127, 1024–1029
11. Chou, D. K., Ilyas, A. A., Evans, J. E., Costello, C., Quarles, R. H., and Jungalwala, F. B. (1986) *J. Biol. Chem.* 261, 11717–11725
12. Ariga, T., Kohriyama, T., Freddo, L., Latov, N., Saito, M., Kon, K., Ando, S., Suzuki, M., Hemling, M. E., Rinehart, K. L., Kusunoki, S., and Yu, R. K. (1987) *J. Biol. Chem.* 262, 848–853
13. Margolis, R. K., Ripellino J. A., Goossen B., Steinbrich R., Margolis R. U. (1987) *Biochem. Biophys. Res. Commun.* 145, 1142–1148
14. Bronner-Fraser, M. (1986) *Dev. Biol.* 115, 44–55
15. Schwarting, G. A., Jungalwala, F. B., Chou, D. K., Boyer, A. M., and Yamamoto, M. (1987) *Dev. Biol.* 120, 65–76
16. Martini, R., Schachner, M., and Brushart, T. M. (1994) *J. Neurosci.* 14, 7180–7191
17. Liedtke, S., Geyer, H., Wuhrer, M., Geyer, R., Frank, G., Gerardy-Schahn, R., Zähringer, U., and Schachner, M. (2001) *Glycobiology* 11, 373–384
18. Martini, R., Xin, Y., Schmitz, B., and Schachner, M. (1992) *Eur. J. Neurosci.* 4, 628–639
19. Kunemund, V., Jungalwala, F. B., Fischer, G., Chou, D. K. H., Keilhauer, G., and Schachner, M. (1988) *J. Cell Biol.* 106, 213–223
20. Voshol, H., van Zuylen, C. W. E. M., Orberger, G., Vliegenthart, J. F. G., and Schachner, M. (1996) *J. Biol. Chem.* 271, 22957–22969
21. Kruse, J., Mailhammer, R., Wernecke, H., Faissner, A., Sommer, I., Goridis, C., and Schachner, M. (1984) *Nature* 311, 153–155
22. Yuen, C-T., Chai, W., Loveless R. W., Lawson, A. M., Margolis, R. U., and Feizi, T. (1997) *J. Biol. Chem.* 272, 8924–8931
23. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) *Dev. Cell* 1, 717–724
24. Dickson, G., Gower, H. J., Barton, C. H., Prentice, H. M., Elsom, V. L., Moore, S. E., Cox, R. D., Quinn, C., Putt, W., and Walsh, F. S. (1987) *Cell* 50, 1119–1130
25. Walsh, F. S., Parekh, R. B., Moore, S. E., Dickson, G., Barton, C. H., Gower, H. J., Dwek, R. A., and Rademacher, T. W. (1989) *Development* 105, 803–811
26. Chou, D. K. H., and Jungalwala, F. B. (1993) *J. Biol. Chem.* 268, 330–336
27. Terayama, K., Oka, S., Seiki, T., Miki, Y., Nakamura, A., Kozutsumi, Y., Takio, K., and Kawasaki, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 6093–6098
28. Bakker, H., Friedmann, I., Oka, S., Kawasaki, T., Nifant'ev, N., Schachner, M., and Mantei, N. (1997) *J. Biol. Chem.* 272, 29942–29946
29. Ong, E., Yeh, J. C., Ding, Y., Hindsgaul, O., and Fukuda, M. (1998) *J. Biol. Chem.* 273, 5190–5195
30. Bierhuizen, M. F. A., and Fukuda, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 9326–9330
31. Simmons, D. L. (1993) in *Cellular Interactions in Development* (Hartly, D., ed) pp. 93–127, Oxford University Press, Oxford
32. Franceschini, I., Angata, K., Ong, E., Hong, A., Doherty, P., and Fukuda, M. (2001) *Glycobiology* 11, 231–239
33. Ong, E., Yeh, J-C., Ding, Y., Hindsgaul, O., Pedersen L. C., Negishi, M., and Fukuda, M. (1999) *J. Biol. Chem.* 274, 25608–25612
34. Kumar, R., Yang, J., Larsen, R. D., and Stanley, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 9948–9952
35. Sarkar, M., Hull, E., Nishikawa, Y., Simpson, R. J., Moritz, R. L., Dunn, R., and Schachter, H. (1991) *Proc. Natil. Acad. Sci. U. S. A.* 88, 234–238
36. Stanley, P., Narasimhan, S., Siminovitch, L., and Schachter, H. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 3323
37. Merkle, R. K., and Cummings, R. D. (1987) *J. Biol. Chem.* 262, 8179–8189
38. Bierhuizen, M. F., Maemura, K., and Fukuda, M. (1994) *J. Biol. Chem.* 269, 4473–4479
39. Obata, K., and Tanaka, H. (1988) *Neurosci. Res.* 6, 131–142
40. Deutscher, S. L., Nuwayhid, N., Stanley, P., Briles, E. I., and Hirschberg, C. B. (1984) *Cell* 39, 295–299
41. Hiraoka, N., Petryniak, B., Nakayama, J. Tsuboi, S., Suzuki, M., Yeh, J-C., Izawa, D., Tanaka, T., Miyasaka, M., Lowe, J. B., and Fukuda, M. (1999) *Immunity* 11, 79–89
42. Nagasawa, K., Inoue, Y., and Tokuyasu, T. (1979) *J. Biochem. (Tokyo)* 86, 1323–1329

43. Carlsson, S. R., Sasaki, H., and Fukuda, M. (1986) *J. Biol. Chem.* **261**, 12787–12795
44. Bélot, F., and Jacquinet J. C. (2000) *Carbohydr. Res.* **326**, 88–97
45. Ujita, M., McAuliffe, J., Hindsgaul, O., Susaki, K., Fukuda, M. N., and Fukuda, M. (1999) *J. Biol. Chem.* **274**, 16717–16726
46. McAuliffe J. C., Fukuda, M., and Hindsgaul, O. (1999) *Bioorg. Med. Chem. Lett.* **9**, 2855–2858
47. Misra, A. K., Fukuda, M., and Hindsgaul, O. (2001) *Bioorg. Med. Chem. Lett.* **11**, 2667–2669
48. Li, F., Wilkins, P. P., Crawley, S., Weinstein, J., Cummings, R. D., and McEver, R. P. (1996) *J. Biol. Chem.* **271**, 3255–3264
49. Carlsson, S. R., Lycksell, P. O., and Fukuda, M. (1993) *Arch. Biochem. Biophys.* **304**, 65–73
50. Tsuzukida, Y., Wang, C. C., and Putnam, F. W. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 1104–1108
51. Baenziger, J., and Kornfeld, S. (1984) *J. Biol. Chem.* **249**, 7270–7281
52. Viitala, J., Carlson, S. R., Siebert, P. D., and Fukuda, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 3743–3747
53. Fukuda, M. (1991) *J. Biol. Chem.* **266**, 21327–21330
54. Yeh, J. C., Ong, E., and Fukuda, M. (1999) *J. Biol. Chem.* **274**, 3215–3221
55. Li, F., Erickson, H. P., James, J. A., Moore, K. L., Cummings, R. D., and McEver, R. P. (1996) *J. Biol. Chem.* **271**, 6342–6348
56. Figarella-Branger, D., Daniel, L., André, P., Guia, S., Renaud, W., Monti, G., Vivier, E., and Rougon, G. (1999) *Am. J. Pathol.* **155**, 1261–1269
57. Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., De Bellard, M. E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994) *Curr. Biol.* **4**, 965–972
58. Bistrup, A., Bhakta, S., Lee, J. K., Belov, Y. Y., Gunn, M. D., Zuo, F. R., Huang, C. C., Kannagi, R., Rosen, S. D., and Hemmerich, S. (1999) *J. Cell Biol.* **145**, 899–910
59. Ujita, M., McAuliffe, J., Schwientek, T., Almeida, R., Hindsgaul, O., Clausen, H., and Fukuda, M. (1998) *J. Biol. Chem.* **273**, 34843–34849
60. Maemura, K., and Fukuda, M. (1992) *J. Biol. Chem.* **267**, 24379–24386
61. Priatel, J. J., Chui, D., Hiraoka, N., Simmons, C. J. T., Richardson, K. B., Page, D. M., Fukuda, M., Varki, N. M., and Marth, J. D. (2000) *Immunity* **12**, 273–283